APPENDIX H: Nobori *et al.* (1996) *Proc. Natl. Acad. Sci.* USA 93:6203-6208.

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Genomic cloning of methylthioadenosine phosphorylase: A purine metabolic enzyme deficient in multiple different cancers

(T-cell acute lymphoblastic leukemia/chromosome 9p/tumor suppressor gene)

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ABSTRACT   5′-Deoxy-5′-methylthioadenosine phosphorylase (methylthioadeno-sine: ortho-phosphate methylthioribosyltransferase, EC 24.2.28; MTAP) plays a role in purine and polyamine metabolism and in the regulation of transmethylation reactions. MTAP is abundant in normal cells but is deficient in many cancers. Recently, the genes for the cyclin-dependent kinase inhibitors p16 and p15 have been localized to the short arm of human chromosome 9 at band p21, where MTAP and interferon α genes (IFNA) also map. Homozygous deletions of p16 and p15 are frequent malignant cell lines. However, the order of the MTAP, p16, p15, and IFNA genes on chromosome 9p is uncertain, and the molecular basis for MTAP deficiency in cancer is unknown. We have cloned the MTAP gene, and have constructed a topologic map of the 9p21 region using yeast artificial chromosome clones, pulse-field gel electrophoresis, and sequence-tagged-site PCR. The MTAP gene consists of eight exons and seven introns. Of 23 malignant cell lines deficient in MTAP protein, all but one had complete or partial deletions. Partial or total deletions of the MTAP gene were found in primary T-cell acute lymphoblastic leukemias (T-ALL). A deletion breakpoint of partial deletions found in cell lines and primary T-ALL was in intron 4. Starting from the centromeric end, the gene order on chromosome 9p21 is p15, p16, MTAP, IFNA, and interferon β gene (IFNB). These results indicate that MTAP deficiency in cancer is primarily due to codeletion of the MTAP and p16 genes.

5′-Deoxy-5′-methylthioadenosine phosphorylase (methylthio-adeno-sine: ortho-phosphate methylthioribosyltransferase, EC 24.2.28; MTAP) is abundant in all normal tissues (1). The substrate for this enzyme, methylthioadenosine (MTA), inhibits the aminopropyltransferases that synthesize polyamines from putrescine and decarboxylated S-adenosylmethionine (2), and also impairs S-adenosylmethionine dependent transmethylation reactions (see ref. 3 for review). MTAP normally prevents the inhibition by cleaving MTA to adenine and 5′-methylthioribose L-phosphate, that are recycled to adenine nucleotides and methionine, respectively (4, 5).

MTAP deficiency is common in human and murine malignant cell lines (1, 6). The abnormality is not confined to tissue culture cells, but is also present in primary leukemias, gliomas, and nonsmall cell lung cancers (7–9). All enzyme negative cell lines lack immunoreactive MTAP (8). In contrast, MTAP-deficient cell lines generated by deliberate mutagenesis and selection contain antigenic enzyme protein (10). Collectively, these results suggested that naturally occurring MTAP deficiency was the result of structural aberrations in the MTAP gene.

Several years ago, the locus for MTAP gene was mapped to the short (p) arm of human chromosome 9 by using somatic cell hybrids (11). Deletions and translocations of chromosome 9p

are frequent in human tumors and are especially common in gliomas (12, 13), melanomas (14), nonsmall cell lung cancers (15, 16), and acute leukemias (17, 18). Recent studies have shown that chromosome 9p21 contains the p16 inhibitor (also designated MTS1) and the p15 inhibitor (also designated MTS2) of cyclin-dependent kinases 4 and 6 (19, 20). The p16 and p15 genes are homozygously deleted in many different malignant cell lines (19, 20) as well as in many primary gliomas (21, 22), acute leukemias (23–25), and pancreatic carcinomas (26).

Malignant cell lines established from malignant tumors with chromosome 9p21 deletions are frequently MTAP deficient (8, 9, 13). In this report, we described the structure and localization of the MTAP gene in relation to the p16 and p15 cyclin-dependent kinase inhibitors. The results indicate that MTAP deficiency in malignancy results from total or partial deletions of the MTAP gene, which is closely linked to the p16 and p15 genes.

MATERIALS AND METHODS

Tumor Cell Lines. Tumor cell lines were obtained from the American Type Culture Collection and from M. O. Diaz (University of Chicago). Hybrid cell line J640-51 was a gift of C. Jones (Eleanor Roosevelt Institute for Cancer Research, Denver) and contains human chromosome 9 on a Chinese hamster background.

Patient Samples. Mononuclear cells were prepared from peripheral blood of patients with T-cell acute lymphoblastic leukemia (T-ALL) enrolled in Pediatric Oncology Group protocol (POG #8862).

Preparation and Analysis of DNA from Cell Lines and T-ALL Patients. Genomic DNA was purified from cell lines and leukemic cells from T-ALL patients by standard methods. The PCR was usually carried out in a total volume of 20 μl, containing 0.1 μg of genomic DNA, 1× PCR buffer (10 mM Tris·HCl, pH 8.3/50 mM KCl/1.5 mM MgCl$_2$/0.01% gelatin), 200 μM of each dNTP, 20 ng each sense and antisense primers, and 0.5 units of Taq DNA polymerase (Boehringer Mannheim). Thirty cycles consisted of 94°C denaturation (1 min), 50 or 55°C annealing (1 min), and 72°C extension (1 min). For PCR amplification of p16 and p15, formamide was added at 5% to the reaction mixture described above, and reactions were cycled 35 times at 94°C for 1 min and 68°C for 3 min. The amplified products were resolved on 2% MetaPhor agarose gels (FMC). For Southern blot analysis, DNA was digested with EcoRI, separated by agarose gel electrophoresis, and

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Table 1.   Oligonucleotides for PCR amplification of the chromosome 9p markers

Marker	Sense primer	Antisense primer	Product size, bp
p15×1	5'-GGAATTCTAGGCTGCGGAATGCGCGAGGAG-3'	5'-ATCATGACCTGGATCGCGCGCCTCCCGAAA-3'	179
71F	5'-GCTTAGTTTTAGAGGGTGAT-3'	5'-AGCAGTTCTTATGAGTGATG-3'	327
p16×1	5'-TGGCTGGTCACCAGAGGGTGGGG-3'	5'-TGCAAACTTCGTCCTCCAGAGTCGCC-3'	300
2F	5'-TGAGAACTAGAGCCTGGAAG-3'	5'-AACCCTCCTTCAAATCTGTA-3'	248
3.21	5'-AGGATGTTGAAGGGACATTG-3'	5'-TGTGTTGTGGACCTCTGTGC-3'	200
MTAP×1	5'-GGGGAGGAAGAGGAGGAGTCAAG-3'	5'-AAGAAGAATCGGGCAGGGCGAACC-3'	237
MTAP×2	5'-ATTGGAATAATTGGTGGAACAGGC-3'	5'-CCAGCAACAGAATGAGAAGTGAT-3'	338
MTAP×3	5'-CAGTCTACCATCAGAGTTCCT-3'	5'-TGGCAAGGAGGACGCAATC-3'	341
MTAP×4	5'-CTCTAGGAGAAAACAGTTGGTG-3'	5'-GACCAGCTACAATAGCCTAAAG-3'	271
MTAP×5	5'-GACCTAGATAAAGTTGACTC-3'	5'-TACACCTTCCAGAAAGACTA-3'	220
MTAP×6	5'-AGTTGTGCATGTGCTAGTAT-3'	5'-ACCCATGCTATATGTGCTTA-3'	328
MTAP×7	5'-AGTTCTAGTAACCTCCAGTG-3'	5'-CTACAGACATGCCTGATTGT-3'	194
MTAP×8	5'-GTGAATATCACTGCCTCCTT-3'	5'-GCTTTTCTTCTGTATTTTAG-3'	273
3.3B	5'-GGGAAGAGACCACACATATA-3'	5'-ACTCATACAGCTTGCTGGTT-3'	238
IFNA8	5'-ACCCTTCTAGATGAATTCTA-3'	5'-GGTCTCATTCCTTACTCTTC-3'	269
IFNB	5'-GGCACAACAGGTAGTAGGCG-3'	5'-GTAACCTGTAAGTCTGTTAAT-3'	592

transferred to Hybond-N⁺ nylon membranes (Amersham). Blots were probed with MTAP cDNA as described (20).

cDNA and Genomic Cloning of the *MTAP* Gene. Briefly, the MTAP protein was purified from rat liver and was microsequenced to obtain the partial sequences of three tryptic peptides. Oligonucleotide primers were synthesized based upon the peptide sequences and were used to PCR amplify a fragment from a human placenta cDNA library (Clontech). The resulting products were subcloned and sequenced. The 5' end of cDNA was obtained by rapid amplification of cDNA end. The cDNA sequence was found to be identical to that recently reported by other investigators (27).

For genomic cloning, human placenta λFIX II (Stratagene) and human chromosome 9-specific (American Type Culture Collection) phage libraries and a cosmid library (Stratagene) were screened with the *PstI–Eco*RI fragment of MTAP cDNA as described (20). After three cycles of screening, DNA was purified from phage and cosmid clones, digested with either *Eco*RI or *Hin*dIII (in conjunction with *Not*I in the case of the λFIX II and cosmid clones), and then was subcloned into pBluescript. If necessary, smaller fragments hybridizing to the MTAP cDNA probe were gel-purified and subcloned.

Yeast Artificial Chromosome (YAC) and P1 Clones. YACs were obtained by PCR screening of human YAC library pools (Research Genetics, Huntsville, AL) with sequence tagged sites (STS) from chromosome 9p [interferon α-8 gene (*IFNA8*), *3.3B, 71F, TC3,* and *1.1*]. Positive YAC clones were grown at 30°C for 2 days with shaking at 2000 rpm in YPD medium [yeast extract (10 g/liter)/bactopeptone (20 g/liter)/2% (wt/vol) dextrose]. Yeast DNA was prepared as described (28).

P1 clones were obtained from Genome Systems (St. Louis) after PCR screening with sets of primers that we provided. The DNA inserts were isolated by alkaline lysis, rescued in plasmids, and subcloned according to the supplier's protocols.

Pulse-Field Gel Electrophoresis (PFGE) Analysis. Agarose plugs containing DNA from cultured cells were prepared by mixing 1% InCert agarose (FMC) in 0.01 M phosphate, 0.15 M NaCl (pH 7.4) (PBS) prewarmed at 42°C with an equal volume of fresh cells suspended in PBS at a density of 2 × 10⁷/ml. The agarose-cell mixture was dispensed immediately into a plug mold in 100 μl aliquots. Plugs were subsequently treated with proteinase buffer [0.5 M EDTA, pH 8.0/1% (wt/vol) sodium lauroyl sarcosinate (Sigma)/2 mg of proteinase K per ml (BRL)] for 48 h at 50°C. Then the plugs were washed three times at room temperature, with sterile TE buffer (10 mM Tris·HCl/1 mM EDTA, pH 8.0) and then twice with TE buffer containing 40 mg of phenylmethylsulfonyl

fluoride per ml at 50°C. After washing with sterile TE buffer, the plug was digested overnight in a 200-μl reaction mixture containing 40 units of restriction enzyme at an appropriate temperature. PFGE was carried out on CHEF-DR II megabase DNA pulsed field electrophoresis system (Bio-Rad) in 0.5 × TBE buffer (90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.0). The ethidium bromide stained DNA was irradiated (300 nm for 1 min) treated with acid (0.25 N HCl for 20 min) and transferred to Hybond-N⁺ nylon membranes for visualization.

Southern blotting was carried out in a hybridization buffer containing 6% polyethylene glycol (PEG 6000; Sigma) as described (29). Following high-stringency washing at 65°C in 0.1× standard saline citrate (SSC) plus 0.1% SDS, membranes were analyzed by autoradiography.

Generation of STSs. STSs were generated by sequencing subcloned plasmids with universal primers (Table 1). Pilot experiments showed that each primer pair produced an amplicon from J640-51 hybrid cells, but not from CHO cells.

RESULTS

Cloning of *MTAP* and an *MTAP* Pseudogene. By screening λFIX II phage and cosmid libraries with *PstI–Eco*RI fragment of MTAP cDNA, two phage clones and one cosmid clone containing exons 5–8 were isolated, and the nucleotide sequences of these four exons and their flanking regions were determined. The *PstI–Hinc*II fragment of cDNA was subsequently used to rescreen the human chromosome 9-specific library, and one clone, λ17-2, was found to contain exons 1–4 (Fig. 1). A separate phage clone (subclone X4 from λMTAP25) contained sequences 91% homologous to exons 2–7, but with stop codons (data not shown, but deposited in GenBank). This clone contained 23 bases matched to the coding sequence of exon 8.

The protein-coding sequence of the *MTAP* gene was interrupted by seven introns (Fig. 2). Exon 1 encodes 11 amino acids and the 5' noncoding region. The sizes of exons 2–7 range from 79 to 240 bp. The last (8th) exon encodes the C-terminal 12 amino acids and the 3' noncoding region. Intron 4 has the SfiI restriction site that was identified in phage clone λMTAP8 (Fig. 1).

Separate DNA fragments containing MTAP cDNA, each exon, and the pseudogene were used for Southern blot analysis of *Eco*RI-digested DNA from human placenta and from YAC clones (data not shown). The size(s) of the *Eco*RI fragments containing each exon and X4 were as follows: 12 kb (exon 1), 2 kb (exon 2), 1.3 kb (exon 3), 4.4 kb (exon 4), 8 kb (exon 5), 0.9 kb (exon 6), 2.7 kb (exon 7), 0.7 kb (exon 8), and 3.5 kb (X4).
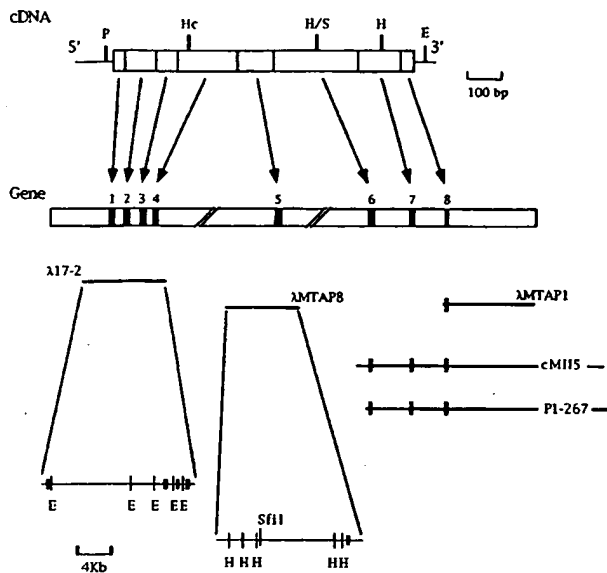
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FIG. 1. The exon–intron organization of the human MTAP gene. The protein-coding regions of the MTAP cDNA are indicated by open boxes. The exons in the MTAP gene are numbered in Arabic and shown by solid blocks. The exact size of the seven introns are indeterminate. More detailed maps of λ17-2 and λMTAP8 were shown at the bottom. λMTAP1, cMII5, and P1-267 are phage, cosmid, and P1 clones, respectively. E, EcoRI; H, HindIII; Hc, HincII; P, PstI; S, SmaI.

The probe X4 detected the 3.5-kb EcoRI fragment in human placenta, but no fragment in J640-51 cells. Moreover, PCR was employed to amplify a 247-bp fragment from the MTAP pseudogene in human placenta, λMTAP25, and J640-51 cells by using primers corresponding to exon 2 and exon 4. The 247-bp fragment was amplified from human placenta and λMTAP25, but not from J640-51 cells (Fig. 3). Taken together, these results indicate that X4 does not map to human chromosome 9 and is a pseudogene.

Analysis of Malignant Cell Lines and Primary T-ALL Samples. Twenty-three MTAP-negative malignant cell lines were analyzed by exon-specific PCR and by Southern blot analysis of EcoRI-digested DNA (Table 2). Eighteen cell lines lack all exons, whereas four cell lines have a deletion breakpoint between exons 4 and 5. We also found partial or total deletions of the MTAP gene in one-third of primary T-ALL samples (A.B., unpublished data). As observed in cell lines, a deletion breakpoint in partial deletion in primary T-ALL samples occurred in intron 4 (Fig. 4C). These results indicate that the main mechanism for MTAP deficiency in malignancy is total or partial deletions of the MTAP gene. Recently, the nucleotide sequences at the breakpoint junctions in two glioma cell lines having deletions of band 9p21 were reported (30). In the A172 cell line, that was found to lack all exons of the MTAP gene and the centromeric members of the IFNA gene cluster, a tandem heptamer repeat was found on either side of the deletion breakpoint junction. The nucleotide sequence from the proximal side of the breakpoint revealed high homology to long interspersed nuclear elements. Although the possible role of sequence overlaps and repetitive sequences in the rearrangement has been well known, it remains to be determined whether or not the same mechanisms reported are involved in the deletions of the MTAP gene in the enzyme-negative cells.

The 7-2 probe, which contains exon 8 of MTAP, detected a 180-kb SfiI band in DNA prepared from enzyme-positive normal lymphocytes (designated BJL) and J640-51 hybrids by PFGE. Except for DHL9, all enzyme deficient cells tested had no band hybridizing to probe 7-2. In T98G, however, a

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cctggtctcgcactgctcactcccgcgcagtgaggttggcacagccaccgctctgtggctcgcttggttc
                                   +1
ccttagtcccgagcgctcgcccactgcagattcctttcccgtgcagacATGGCCTCTGGCACCACCACTA      EXON 1
                                                  M  A  S  G  T  T  T
    10                                                20
CCGCCGTGAAGgtgagatga..........tgctcttagATTGGAATAATTGGTGGAACAGGCCTGGATG      EXON 2
T  A  V  K          intron 1          I  G  I  I  G  G  T  G  L  D
                   30                                  40
ATCCAGAAATTTTAGAAGGAAGAACTGAAAAATATGTGGATACTCCATTTGGCAAGgttaatatc.....
D  P  E  I  L  E  G  R  T  E  K  Y  V  D  T  P  P  G  K        intron 2
                                      50
......tgcatgcagCCATCTGATGCCTTAATTTTGGGGAAGATAAAAAAATGTTGATTGCGTCCTCCTTG      EXON 3
                P  S  D  A  L  I  L  G  K  I  K  N  V  D  C  V  L  L
    60                                                70
CAAGgtatggta........cttccatagGCATGGAAGGCAGCACACCATCATGCCTTCAAAGGTCAACT      EXON 4
A  R         intron 3          H  G  R  Q  H  T  I  M  P  S  K  V  N
               80                              90
ACCAGGCGAACATCTGGGCTTTGAAGGAAGAGGGCTGTACACATGTCATAGTGACCACAGCTTGTGGCTC
Y  Q  A  N  I  W  A  L  K  E  E  G  C  T  H  V  I  V  T  T  A  C  G  S
        100                               110
CTTGAGGGAGGAGATTCAGCCCGGCGATATTGTCATTATTGATCAGTTCATTGACAGgtaagcagt....
L  R  E  E  I  Q  P  G  D  I  V  I  I  D  Q  F  I  D  R
               120                              130
.........attttgtagGACCACTATGAGACCTCAGTCCTTCTATGATGGAAGTCATTCTTGTGCCAGA      EXON 5
intron 4          T  T  H  R  P  Q  S  P  Y  D  G  S  H  S  C  A  R
                  140                               150
GGAGTGTGCCATATTCCAATGGCTGAGCCGTTTTGCCCCAAAACGAGAGAGgtgtgtagt..........
G  V  C  H  I  P  M  A  E  P  F  C  P  K  T  R  E        intron 5
                  160                              170
cttttctagGTTCTTATAGAGACTGCTAAGAAGCTAGGACTCCGGTGCCACTCAAAGGGGACAATGGTCA      EXON 6
         V  L  I  E  T  A  K  K  L  G  L  R  C  H  S  K  G  T  M  V
                     180                              190
CAATCGAGGGACCTCGTTTTAGCTCCCGGGCAGAAAGCTTCATGTTCCGCACCTGGGGGGCGGATGTTAT
T  I  E  G  P  R  F  S  S  R  A  E  S  F  M  F  R  T  W  G  A  D  V  I
                  200                               210
CAACATGACCACAGTTCCAGAGGTGGTTCTTGCTAAGGAGGCTGGAATTTGTTACGCAAGTATCGCCATG
N  M  T  T  V  P  E  V  V  L  A  K  E  A  G  I  C  Y  A  S  I  A  M
                  220                               230
GGCACAGATTATGACTGCTGGAAGGAGCACGAGGAAGCAgtaggtgga........tttctctagGTTTC      EXON 7
G  T  D  Y  D  C  W  K  E  H  E  E  A        intron 6          V  S
                  240                               250
GGTGGACCGGGTCTTAAAGACCCTGAAAGAAAACGCTAATAAAGCCAAAAGCTTACTGCTCACTACCATA
V  D  R  V  L  K  T  L  K  E  N  A  N  K  A  K  S  L  L  L  T  T  I
                  260                               270
CCTCAGATAGGGTCCACAGAATGGTCAGAAACCCTCCATAACCTGAAGgtaatgtgc........tcctt
P  Q  I  G  S  T  E  W  S  E  T  L  H  N  L  K        intron 7
                  280
tcagAATATGGCCCAGTTTTCTGTTTTATTACCAAGACATTAAAGTA GCATGGCTGC CCAGGAGAAAA      EXON 8
    N  M  A  Q  F  S  V  L  L  P  R  H  *
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FIG. 2. The DNA sequences of the protein-coding exons and their flanking regions in the human MTAP gene. The nucleotide sequences of eight exons are shown in uppercase letters, while those of flanking sequences are shown in lowercase letters. The sizes of exons 2–7 are 87, 79, 158, 103, 240, and 123 nucleotides, respectively. The deduced peptide sequence is shown below the coding exons and is numbered from the first methionine residue. The translation termination codon TAA is denoted by an *.
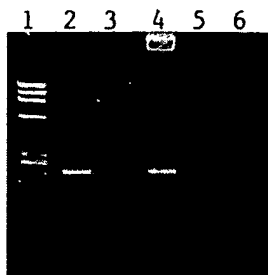
FIG. 3. PCR analysis with primers from exons 2 and 4. PCR amplification was performed as described by using a sense primer (5'-ATATGTGGATACTCCATTTGGCAA-3') from exon 2 and an antisense primer (5'-CTGATCAATAATGACAATATCGCC-3') from exon 4. Lanes: 1, DNA size marker (HaeIII digests of φX174 DNA); 2, human placenta; 3, J640-51; 4, λMTAP25; 5, Chinese hamster ovary cells; 6, no template.

rearrangement caused a shift in size of the normal 180-kb fragment to 110 kb (Fig. 4A).

In Southern blots, the 7-2 probe hybridized to the 2-kb HindIII band in enzyme-positive malignant cell lines as well as in human placenta. No band was detected in enzyme-negative malignant cell lines except for DHL9 (Fig. 4B). However, when EcoRI-digested DNAs from MTAP deficient cells were probed with total MTAP cDNA, at least one band was observed due to hybridization to the MTAP pseudogene (Fig. 4C). Since exon 8 is always deleted in MTAP-deficient cells, MTAP deficiency can be diagnosed by Southern blotting with

Table 2. Deletions of the MTAP exon in MTA phosphorylase-deficient cell lines

Cell line	MTAP exon							
	1	2	3	4	5	6	7	8
Glioma								
A-172	−	−	−	−	−	−	−	−
H4	−	−	−	−	−	−	−	−
Hs 683	−	−	−	−	−	−	−	−
U-138MG	−	−	−	−	−	−	−	−
U-87MG	−	−	−	−	−	−	−	−
Breast cancer								
MCF-7	+	+	+	+	−	−	−	−
MDA-MB-231	−	−	−	−	−	−	−	−
Leukemia								
BLIN-1	−	−	−	−	−	−	−	−
CEM	+	+	+	+	−	−	−	−
DHL-9	+	+	+	+	+	+	+	+
HSB-2	+	+	+	+	−	−	−	−
Jurkat	−	−	−	−	−	−	−	−
K-562	−	−	−	−	−	−	−	−
K-T1	−	−	−	−	−	−	−	−
NALL-1	−	−	−	−	−	−	−	−
Lung cancer								
A549	+	+	+	+	−	−	−	−
SK-LU-1	−	−	−	−	−	−	−	−
SW-900	−	−	−	−	−	−	−	−
H292	−	−	−	−	−	−	−	−
Melanoma								
Hs294T	−	−	−	−	−	−	−	−
Malme-3M	−	−	−	−	−	−	−	−
Bladder								
carcinoma								
RT4	−	−	−	−	−	−	−	−
UM-UC-3	−	−	−	−	−	−	−	−

Homozygous deletions of each MTAP exon were determined by PCR analysis and were confirmed by Southern blotting of EcoRI-digested DNA with the MTAP cDNA probe. +, Presence of DNA; −, homozygous loss.

probe 7-2. However, DNA may not be isolated from primary tumor tissues in a sufficient amount to perform Southern blot analysis. PCR assays for each exon, especially for exon 8, will be an alternative method for detection of MTAP deficiency.

Previous results suggested that p16-deficient T98G glioma cells have a deletion in the region between the MTAP and IFNA gene loci (13, 20). Based upon these previous findings, we have identified and localized the p16 gene between these two loci (20). This issue was reexamined by PFGE of SfiI-digested DNA from YACs.

Characterization of YAC Clones. To construct a more detailed physical map of the 9p21 region encompassing the MTAP gene, the p16 and p15 genes, and the IFNA gene cluster, a human YAC library was screened. Eight YAC clones ranging from 200 to 1400 kb were obtained with STSs IFNA8, 3.3B, 71F, TC3, and 1.1, followed by further analysis with other STSs (Table 3). YAC 802B11 was the most informative isolate. It contained STSs 1.1 (MTAP exon 4), 3.3B, IFNA8, and IFNB, but was negative with STSs TC3 (MTAP exon 5) through p15x1 (p15 exon 1). Pulsed field gel analysis of YAC 802B11 showed a 120-kb SfiI fragment with the MTAP cDNA probe, 320 and 160-kb SfiI fragments with the 3.3B probe, and three SfiI fragments (320, 120, and 80 kb) with the IFNA probe. The SfiI fragments detected with the 3.3B and IFNA probes were identical to those observed in YACs 760C6, 760C7, and 761A5. In these three YACs, which contain all exons of the MTAP gene, the MTAP cDNA probe hybridized to both the 150- and 120-kb SfiI fragments. However, only the 120-kb fragment was detected in YAC 802B11 that contains exons 1–4 of the MTAP gene (Fig. 5). The first four exons in the 120-kb fragment are separated from the last four exons by a SfiI site in intron 4 (Fig. 2). This hybridization pattern is different from that observed in normal lymphocytes, in which the MTAP cDNA probe mainly hybridized to 180- and 250-kb SfiI fragments. The 250-kb SfiI fragment was detectable even in enzyme-negative cell lines, but was absent in the YACs containing MTAP exons. The SfiI blot shown in Fig. 4A was also reprobed with the X4 probe. All enzyme positive and negative cells, except for J640-51, had the 250-kb SfiI fragment (data not shown). Thus, in common with the signal detected by MTAP cDNA in Southern blots of enzyme-negative cells (Fig. 4C), this 250-kb fragment was attributable to cross-hybridization of the probe to the MTAP pseudogene. The observed difference in the size of SfiI fragment containing exons 5–8 between genomic and YAC DNA (Figs. 4A and 5) was found to be artifactual, since the same 150-kb SfiI fragment was detected in normal lymphocytes and YAC 760C6 separated on the same pulsed-field gel following SfiI digestion. These results confirm that the first four exons of the MTAP gene reside in the 120-kb fragment and the last four exons in the 150-kb fragment.

DISCUSSION

The structural gene for MTAP on chromosome 9p is ≈100 kb telomeric to the p16/MTS1 gene (20) and contains eight exons and seven introns. Depending on cell type, p16-deficient cancers have deleted all or part of MTAP at various frequencies (40% in melanomas, 57% in nonsmall cell lung cancers, 71% in gliomas, and 78% in leukemias) (unpublished data). No cancers have been found with MTAP deficiency without homozygous deletions of p16. These results suggest that the loss of MTAP in malignant cells is due to linkage between the MTAP and p16 genes.

MTAP deletions are difficult to detect by Southern blotting and PCR without information on the sequence and intron–exon structure of the genomic MTAP gene, because the MTAP pseudogene can produce a positive result. Although preliminary Southern blotting experiments lead us to believe that MTAP was centromeric to p16 (20), detailed analysis of YAC clones, and of DNA separated in pulse-field gels, refuted this
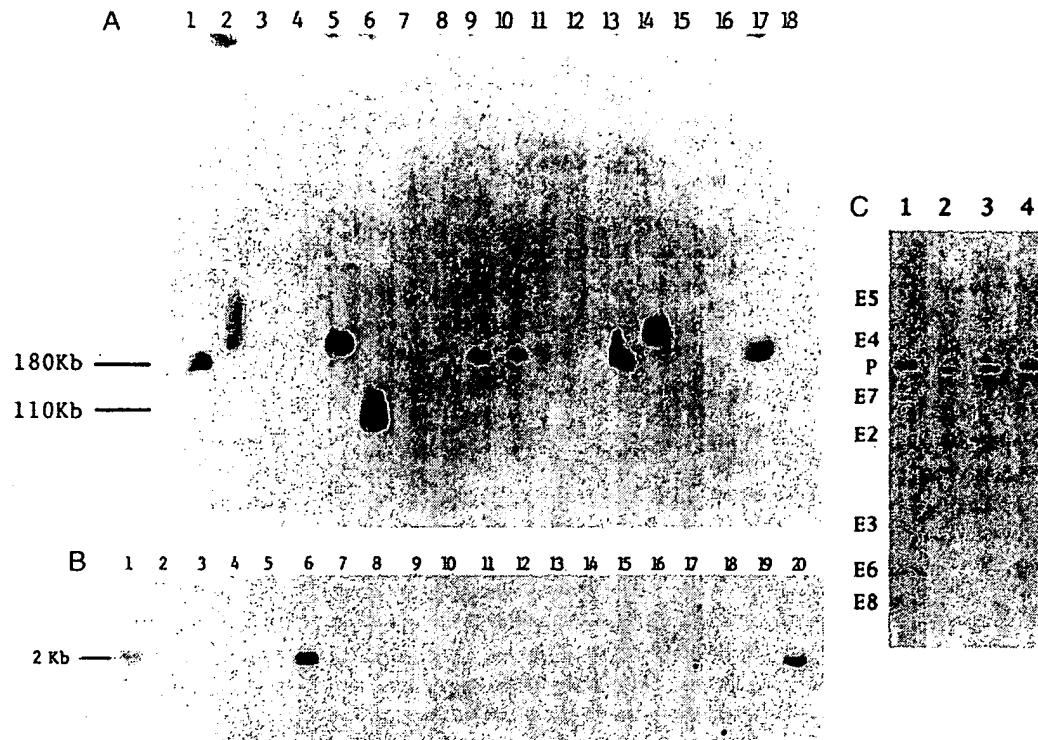
FIG. 4. DNA analysis of the cell lines and primary T-ALL. (A) Pulse-field gel analysis. SfiI digests of DNAs from cell lines including normal lymphocytes and MTAP positive and negative malignant cell lines were fractionated by PFGE and then hybridized with the 7-2 probe containing exon 8 of the MTAP gene. Lanes: 1, normal lymphocytes; 2, J640-51; 3, U-87MG; 4, U-138MG; 5, U-373MG; 6, T98G; 7, H4; 8, Hs683; 9, CALU-1; 10, CALU-6; 11, A-549; 12, SK-LU-1; 13, SK-Mes-1; 14, T24; 15, UM-UC-3; 16, RT4; 17, RPMI-7951; 18, Malme-3M. Lanes 1, 2, 5, 6, 9, 10, 13, 14, and 17 were MTAP-positive cells, whereas lanes 3, 4, 7, 8, 11, 12, 15, 16, and 18 were MTAP-negative cells. (B) Southern blot analysis. HindIII digests of DNAs were separated and hybridized with the 7-2 probe. Lanes: 1, human placenta; 2, NALL-1; 3, HSB2; 4, K-T1; 5, CEM; 6, DHL9; 7, BLIN1; 8, K562; 9, U-87MG; 10, U-138MG; 11, A172; 12, H4; 13, Hs 683; 14, A549; 15, SK-LU-1; 16, UM-UC-3; 17, RT4; 18, MCF-7; 19, Malme-3M; 20, T98G. All lanes but lanes 1 and 20 were MTAP-negative cells. (C) Representative Southern blot analysis of primary T-ALL. DNAs were digested with EcoRI and probed with MTAP cDNA. Although exon 1 was not detected in these samples by Southern blotting, three samples (lanes 1-3) were tested positive with PCR assay for exon 1. Lanes: 1 and 4, T-ALL samples with all intact exons; 2, T-ALL with deletions of exons 4-5; 3, T-ALL with total deletions. E2-8, MTAP exons 2-8; P, a pseudogene.

supposition. Furthermore, some malignant cell lines have homozygous deletions of both p16 and MTAP, but retain an intact p15 gene. Thus, the correct gene order on human chromosome 9p is p15-p16-MTAP-IFNA from centromeric to telomeric. Accordingly, the deleted region in T98G is the region containing p16, centromeric to MTAP, but not the previously proposed region between MTAP and IFNA gene loci.

If the loss of MTAP is due solely to linkage to p16, the abnormality should have the same frequency in p16-deficient cancers arising from different cell types. More than 70% of homozygous p16 deletions in gliomas, and 50% of the deletions in T cell leukemias, include MTAP (20). In contrast, MTAP deficiency is uncommon in melanomas with p16 deletions (20). A possible explanation for the difference is that a second gene on chromosome 9p confers a survival advantage to p16-deficient gliomas and leukemias, but not to melanomas. The second gene is unlikely to be IFNA, since many p16 deficient cell lines have an intact IFNA gene cluster.

Deletions of the p16 gene in some primary cancers are apparently much more frequent than intragenic mutations (21-26). It is conceivable that structural features of the p15/p16/MTAP loci facilitate recombination, perhaps due to repetitive sequences. Deletions would also be favored if the loss of two genes produced a greater survival advantage than a deficiency of p16 alone (21).

Table 3. Analysis of YAC clones by PCR and Southern blot hybridization

YAC	Size, kb	p15×1	71F	p16×1	2F	3.21	ex8	ex7	ex6	ex5	ex4	ex3	ex2	ex1	3.3B	IFNA8	IFNB
759B7	900	−	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−
760C6	1200	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
760C7	1200	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
802B11	1400	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+
761A5	1100	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
735B2	200	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−
735H8	500	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−
660H9	840	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−

Homozygous deletions of each marker in YAC clones were detected by PCR and Southern blot analyses. IFNB is telomeric and p15×1 is centromeric. Markers p15×1, p16×1, and ex1-8 are exon 1 of the p15 gene, exon 1 of the p16 gene, and exons 1-8 of the MTAP gene. Other markers were described in ref. 20. +, Presence of DNA; −, homozygous loss.
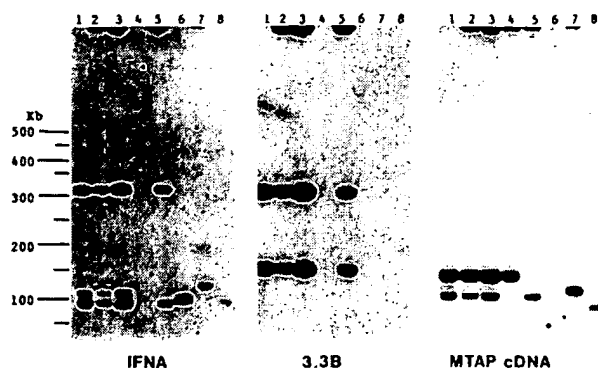
FIG. 5. Pulse-field gel analysis of YAC clones with probes *IFNA*, 3.3B, and MTAP cDNA. SfiI digests of YAC DNA were fractionated by PFGE and then hybridized with the indicated probe. The probe IFNA is cDNA and the probe 3.3B is the 1.4-kb *Eco*RI fragment containing an internal SfiI site derived from the *IFNA*-positive YAC clone. Lanes: 1, 761A5; 2, 760C6; 3, 760C7; 4, 660H9; 5, 802B11; 6, 735H8; 7, 759B7; 8, 735B2.

MTAP deficiency is a simple marker for a chromosome 9p deletion because normal cells contain abundant enzyme protein. Normal cells reconvert MTA to adenine nucleotides and methionine, whereas MTAP-deficient tumor cells have lost these salvage pathways. As such, cancers with deletions of *MTAP* gene may be especially susceptible to chemotherapeutic regimens that interfere with purine or methionine utilization (1, 9). In addition, MTA, the substrate for MTAP, is a natural inhibitor of S-adenosylmethionine-dependent transmethylation reactions (3). It is conceivable that even a transient disturbance in DNA methylation could promote the progress of a malignant tumor.

The assessment of MTAP deficiency in gliomas, lung cancers, leukemias, and other cancers could have diagnostic and prognostic value. MTAP deficiency is mainly caused by partial or total deletions of the *MTAP* gene not only in cell lines but also in primary tumors. Our work presented herein will facilitate the development of molecular diagnosis of MTAP deficiency and understanding of molecular mechanisms of this deficiency.

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